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(54) Title: IDENTIFICATION AND USE OF ANTIVIRAL COMPOUNDS

(57) Abstract

The present invention relates to the identification of host cell proteins that interact with viral proteins required for virus replication, and high throughput assays to identify compounds that interfere with the specific interaction between the viral and host cell protein. Interfering compounds that inhibit viral replication can be used therapeutically to treat viral infection. The invention is based, in part, on the Applicants' discovery of novel interactions between proteins of the influenza virus and human host cell proteins. One of these host cell proteins, referred to herein as NPI-1, interacts with influenza virus protein NP, and may be an accessory protein required for replication of influenza virus. Another of these host cell proteins, referred to herein as NS1I-1, interacts with influenza virus protein NS$_1$. Compounds that interfere with the binding of the host cell and viral proteins, and inhibit viral replication can be useful for treating viral infection *in vivo*.

IDENTIFICATION AND USE OF ANTIVIRAL COMPOUNDS

This application is a continuation-in-part of co-pending application Serial No. 08/246,583, filed May 20, 1994, which is hereby incorporated by reference in its entirety.

1. INTRODUCTION

The present invention relates to the identification of new cellular targets for viral intervention, the identification of antiviral compounds that act on the new targets, and the therapeutic use of such antiviral compounds.

2. BACKGROUND OF THE INVENTION

Influenza A virus is a negative strand RNA virus belonging to the orthomyxovirus family. The genome of the virus consists of 8 segments and encodes 10 polypeptides. Experimental evidence generated in the laboratory of Scholtissek indicates that the nucleoprotein (NP) is a major determinant of species specificity of influenza viruses (Scholtissek, et al., 1985, Virology 147: 287-294). Phylogenetic analysis divides NP genes into two families: one containing NPs predominantly of avian origin, and one containing those of human origin (Bean, 1984, Virology 133:438-442; Buckler-White & Murphy, 1986, Virology 155: 345-355; Gammelin, et al., 1989, Virology 170:71-80; Scholtissek, et al., 1985, supra). The human virus A/HK/1/68 and viruses having genetically related NPs cannot rescue mutants of the avian virus A/FPV/Rostock/1/34 with ts defects in the NP following double infection of chicken embryo fibroblasts (CEF) at 40°C (Scholtissek, et al., 1985, supra; Scholtissek, et al., 1978, Virology 91: 79-85). However, the human viruses which failed to rescue the

ts mutants on CEF cells were able to do so on Madin-
Darby canine kidney(MDCK) cells (Scholtissek, et al.,
1978, *supra*). Additionally, A/HK/1/68 virus and
A/FPV/Rostock/1/34 virus reassortants containing the
A/HK/1/68 virus-derived NP replicate in MDBK cells but
5 not in CEFs (Scholtissek, et al., 1978, *supra*). The
host-specific rescue of FPV ts mutants and the host
restriction of A/HK/1/68 virus reassortants suggest
that a factor(s) of host origin, which differs between
mammalian and avian cells, is responsible for this
10 phenomenon, and that this factor may interact with the
influenza A virus NP. However, heretofore, no host
protein has been identified.

Replication and transcription of influenza virus
RNA requires four virus encoded proteins: the NP and
15 the three components of the viral RNA-dependent RNA
polymerase, PB1, PB2 and PA (Huang, et al., 1990,
J. Virol. 64: 5669-5673). The NP is the major
structural component of the virion which interacts
with genomic RNA, and is required for antitermination
20 during RNA synthesis (Beaton & Krug, 1986, Proc. Natl.
Acad. Sci. USA 83:6282-6286). NP is also required for
elongation of RNA chains (Shapiro & Krug, 1988,
J. Virol. 62: 2285-2290) but not for initiation
(Honda, et al., 1988, J. Biochem. 104: 1021-1026).
25 NS1 is a major non-structural protein
expressed by influenza A viruses in infected cells,
whose role in infection is not clear. Studies of
viruses carrying temperature-sensitive NS1 alleles
point to a regulatory role for NS1 in viral gene-
30 expression and/or replication (Wolstenholme, et al.,
1980, J. Virol. 35:1-7; Koennecke, et al., 1981,
Virol. 110:16-25; Hatada, et al., 1990, J. Gen. Virol.
71: 1283-1292), which is also consistent with its
preferentially nuclear accumulation (Greenspan, et
35 al., 1988, J. Virol. 62: 3020-3026). Its expression

●   ●

has been shown to interfere with cellular functions in
a variety of ways. (Fortes, et al., 1994, EMBO J. 13:
704-712; Qiu & Krug, 1994, J. Virol. 68: 2425-2432;
Lu, et al., 1994, Genes Dev. 8: 1817-1828). These
effects have been suggested to be mediated through
5   NS1's observed interactions with a variety of RNA's,
including single- and double-stranded influenza vRNA
(Hatada & Fukuda, 1992, J. Gen. Virol. 73: 3325-3329;
Hatada, et al., 1992, J. Gen Virol. 73: 17-25), poly-
adenosine RNA (Qiu & Krug, 1994, supra), and
10 spliceosomal U6 RNA (Lu, et al., 1994, supra).
Despite these studies involving the interaction of NS1
with various RNAs, no host proteins that interact with
NS1 during infection have previously been identified
or characterized.
15   Little is known about host cell functions which
contribute to the intracellular replication of
influenza viruses, and cellular factors have not been
characterized which directly interact with the viral
proteins, much less cellular factor/viral interactions
20 that can be used as targets for therapeutic
intervention.

3.   SUMMARY OF THE INVENTION
The present invention relates to the
25 identification of host cell proteins that interact
with viral proteins required for virus replication,
and high throughput assays to identify compounds that
interfere with the specific interaction between the
viral and host cell protein. Interfering compounds
30 that inhibit viral replication can be used
therapeutically to treat viral infection.
The invention is based, in part, on the
Applicants' discovery of a novel interaction between
influenza viral proteins, such as NP and NS1, and
35 human host cell proteins referred to herein as NPI-1,

NPI-2, NPI-3, NPI-4, NPI-5, NPI-6, and NS1I-1,
respectively. The host cell proteins such as NPI-1
and NS1I-1 may be accessory proteins required for
replication of influenza virus. Compounds that
interfere with the binding of the host cell and viral
5 proteins and inhibit viral replication can be useful
for treating viral infection in vivo.

4. DESCRIPTION OF THE FIGURES

FIG. 1A and 1B: The interactive trap system, as
10 used in the identification of NP-interacting proteins.
FIG. 1A: R100 contains the reporter gene LexAop-LEU2
and a transcriptionally inactive LexA-NP fusion
protein (left). Library proteins are synthesized in
R100 transformants in media containing galactose. If
15 the library protein does not interact with the LexA-NP
fusion protein, then the LEU2 gene is not transcribed
(middle). If the library protein does interact with
the LexA-NP fusion protein, then the LEU2 gene is
transcriptionally active, and the cell forms a colony
20 on leu⁻ medium (right). FIG. 1B: The pLexA-NP bait
plasmid used in the interactive trap. The coding
region of influenza A/PR/8/34 virus nucleoprotein (NP)
was subcloned into the *EcoR1* and *Sal I* restriction
sites of pEG202. This construction encodes a fusion
25 protein which includes 202 amino acids of LexA and the
entire coding region of NP (498 amino acids) separated
by 3 amino acids encoded by polylinker sequences
derived from the cloning process.

FIG. 2. Nucleotide sequence of NPI-1 cDNA and
30 deduced protein sequence. The coding sequence starts
at nucleotide 1 and ends at nucleotide 1581. The 5'
terminus of the library clone is indicated by an
asterisk. Regions complementary to nested reverse
transcription and 5'RACE primers are underlined.
35

FIG. 3. Comparison of NPI-1 and SRP1. Vertical lines indicate identity; colons and periods indicate conservative changes (Deveraux et al., 1984, Nucl. Acids Res. 12: 387-395). 42 amino acid ARM repeats are aligned vertically according to Peifer et al., 1994,
5 Cell 76: 789-791. For a complete comparison of SRP1 to other ARM repeat containing proteins, see Peifer et al., 1994, supra. The ARM consensus sequence is indicated at the bottom; "+" indicates K,R, or H; "-" indicates D or E; "~" indicates a gap. Since other
10 residues are conserved within the repeats of NPI-1 and SRP1, a consensus sequence derived from only these two proteins is also shown.

FIG. 4. GST-NPI-1 binds to NP in vitro. GST (lanes 1,5,6) and GST-NPI-1 (lanes 2,3,7,8) were
15 expressed in bacteria and precipitated from cell lysates on glutathione agarose beads. The complexed beads were then incubated with partially purified influenza virus NP and polymerase preparations (Pol/NP) as indicated. Precipitated proteins were
20 fractionated on a 12.5% SDS polyacrylamide gel, and either stained with Coomassie blue (lanes 1 to 3), or immunoblotted using the monoclonal antibody HT103 directed against the viral nucleoprotein (lanes 4 to 8). Unprecipitated Pol/NP was separated in lane 4.
25 M, protein molecular weight markers; *, GST-NPI-1 fusion protein; arrows indicate major fusion protein degradation products.

FIG 5. Immunoblot of total cellular proteins using polyclonal rabbit sera against NPI-1. Total
30 cell lysates and cytoplasmic cell extracts from HeLa and MDBK cell lines were separated by SDS-PAGE, transferred to nitrocellulose, immunoblotted with anti-NPI-1 sera, and developed by ^{125}I-protein A. Each lane contains protein from 1 x 10^5 cells.
35

FIG 6. NP is co-immunoprecipitated from influenza A virus infected cells by antisera against NPI-1. Infected HeLa cell proteins were labeled with ^{35}S-methionine and ^{35}S-cysteine, and total cell lysates were made as described in the text. Complexes of
5 NPI-1 and NP were precipitated using anti-NPI-1 sera. Precipitated proteins were then fractionated by SDS-PAGE and detected by autoradiography.

FIGS 7-11. Partial DNA sequences of isolated coding regions of five different proteins that
10 interact with the NP of influenza A, as detected using the interactive trap system in yeast. The proteins whose sequences are provided are as follows:

FIG. 7: Partial nucleotide sequence of NPI-2.

FIG. 8: Partial nucleotide sequence of NPI-3.
15 FIG. 9: Partial nucleotide sequence of NPI-4.

FIG. 10: Partial nucleotide sequence of NPI-5.

FIG. 11: Partial nucleotide sequence of NPI-6.

Fig. 12. Nucleotide sequence of the NS1I-1 gene and the encoded amino acid sequence of the NS1I-1
20 protein. The sequence of 2572 bp was determined by dideoxy sequencing of two overlapping clones. The first clone, pK5, was isolated from the yeast library and contains the HeLa cell cDNA comprising nucleotide positions 791 to 2572. The second clone, pRACENS1I-1,
25 resulted from the 5'RACE procedure used to obtain cDNA derived from the 5'-end of NS1I-1 mRNA, and comprises nucleotide positions 1 to 944.

Fig. 13. Northern blot analysis of HeLa cell poly(A)-RNA using an NS1I-1-specific probe.
30 Fig. 14. Co-precipitation of NS1 protein from extracts of A/WSN/33-infected MDCK cells by GST-NS1I-1 and glutathione sepharose. Monolayers of MDCK cells were either infected with influenza A/WSN/33 virus at an m.o.i. of 10 or mock-infected, and labeled with ^{35}S-
35 methionine and cysteine from 5 to 6 hours p.i.

Proteins were extracted and used for binding to
glutathione sepharose coated with GST-NS1I-1 (lanes 3
and 8) or GST-protein (lane 6). As controls, extracts
were immunoprecipitated with α-NS1 (lane 2), α-M1
(lane 4), or non-immune serum (lane 5). Proteins were
5   analyzed by SDS gel electrophoresis and fluorography.
Aliquots of the total extracts corresponding to 10%
used for the glutathione agarose precipitations are
shown (lanes 1 and 7). The positions of virus
proteins and molecular weight markers are indicated to
10   the left.

        Fig. 15. GST-NS1I-1 co-precipitates NS1 proteins
of influenza A and B virus strains. Extracts of
^{35}S-labeled MDCK cells infected with the influenza
viruses A/duck/Alberta/76 (Panel A), A/turkey/Oregon
15   (Panel B), A/Beijing/32/92 (Panel C), A/Berkeley/1/68
(Panel D), and B/Lee/40 (Panel E) were prepared and
used in precipitations of viral proteins by
glutathione-sepharose coated with GST-NS1I-1 (lanes
"GST-K5") or GST-protein (lanes "GST") as described in
20   Fig. 14. In addition, viral proteins were
immunoprecipitated using α-NS1-, α-M1- or non-immune
serum (lanes "α-NS1", "α-M1", "NI", respectively).
Analysis was by SDS gel electrophoresis and
fluorography. Aliquots of the total extracts
25   corresponding to 10% (Panels C and E) or 6.7% (Panels
A, B, and D), respectively, are also shown (lanes
"T"). The positions of viral proteins are indicated
to the right.

30   5. DETAILED DESCRIPTION OF THE INVENTION
        The present invention relates to the
identification of host cellular proteins that interact
with viral proteins important to viral replication and
infection; the identification of compounds that
35   interfere with the specific interaction of the host

- 7 -

cell and viral proteins; and the evaluation and use of
such compounds as antivirals in the treatment of viral
infections in animals, including humans.

The invention is described in this section and in
the examples, below for the identification and
5 inhibition of interactions between human host cell
proteins and influenza viral proteins. For clarity of
discussion, particular detail is provided for the
isolation of two particular host cell proteins. The
first such protein is nucleoprotein interactor 1
10 (NPI-1), a human cell protein that interacts with the
influenza virus NP protein. The NPI-1 gene and
protein, and the protein's interaction with NP protein
are described in detail in the example in Section 6,
below. Other host cell proteins which interact with
15 the NP protein include, but are not limited to, NPI-2,
NPI-3, NPI-4, NPI-5, and NPI-6, and are also
described, below. Since the interactions between NP
and the NPI-1 through NPI-6 host cell proteins have
never before been identified, they provide novel
20 targets for antiviral treatment and serve as excellent
models for detailing the aspects of the invention.
However, the principles may be analogously applied to
the identification of other host cell proteins that
interact with any of the four influenza virus proteins
25 (PA, PB1, PB2, in addition to NP) required for viral
RNA replication.

Particular detail is also provided in the example
in Section 7, below, for the identification of
nonstructural protein 1 interactor 1 (NS1I-1). NS1I-1
30 is a human cell protein that interacts with the
influenza virus NS1 protein. This interaction also
has never before been described, and, therefore,
provides yet another novel target for antiviral
treatment. The present invention also contemplates
35 identifying interactions between host cell proteins

and other viral proteins (in addition to NS_1) required
for infection, such as, in the case of influenza
virus, NS_2 HA, NA, M_1, and M_2 proteins.

The principles may also be analogously
applied to other RNA viruses, including but not
5   limited to paramyxoviruses, such as parainfluenza
viruses, measles viruses, respiratory syncytial virus,
bunyviruses, arena viruses, the orthomyxo-like insect
virus called Dhori, etc. The host cell proteins so
identified may include completely novel proteins, or
10   previously described proteins that have not yet been
shown to interact with viral proteins. Any method
suitable for detecting protein-protein interactions
may be employed for identifying novel viral-host
protein interactions, and are considered within the
15   scope of the present invention. For example, some
traditional methods are co-immunoprecipitation,
crosslinking and copurification through gradients or
chromatographic columns. Newer methods result in the
simultaneous identification of the genes coding for
20   the protein interacting with a target protein. These
methods include probing expression libraries with
labeled target protein in a manner similar to antibody
probing of λgt11 libraries. One such method which
detects protein interactions *in vivo*, the yeast
25   interactive trap system, was successfully used as
described herein to identify the host cell proteins
NPI-1 through NPI-6, and NS1I-1, described herein, and
is described in detail for illustration only and not
by way of limitation.
30   The host cell/viral protein interactions
identified are considered targets for antiviral
intervention. Assays, such as the ones described
herein, can be used to identify compounds that
interfere with such interactions. The compounds so
35   identified which inhibit virus infection, replication,

assembly, or release can be used as antivirals. In
accordance with the invention, a given compound found
to inhibit one virus may be tested for antiviral
activity against a wide range of different viruses
that have analogous dependencies on host cell
5 proteins, including but not limited to
paramyxoviruses, such as parainfluenza viruses,
measles viruses, respiratory syncytial virus,
bunyviruses, arena viruses, the orthomyxo-like insect
virus called Dhori, etc.
10   Elucidation of the roles of the interacting
proteins will lead to identifying other viruses as
targets for intervention. For example, we have found
that NPI-1 is important to the import of viral nucleic
acid-protein complex into the nucleus of the host
15 cell. Therefore, methods described below that disrupt
this process, through interfering with the activity of
NPI-1, for example, may be effective in treating
viruses with nuclear phases, in addition to those
viruses listed above. Such additional viruses
20 include, but are not limited to, human
immunodeficiency virus (HIV), members of the herpes
virus family, and adenoviruses.
    The various aspects of the invention are
described in the subsections below with specific
25 reference to host cell proteins that interact with NP
(NPI-1 through NPI-6) and NS1 (NS1I-1), with
particular emphasis on NPI-1; however, the invention
is not limited to NPI-1 and encompasses any viral/host
cell protein interactions as targets for therapeutic
30 intervention.

    5.1. IDENTIFICATION OF HOST CELL PROTEINS
          THAT INTERACT WITH VIRAL PROTEINS
          REQUIRED FOR REPLICATION

35

The previously unidentified gene for the host
cell protein NPI-1 was cloned based on its ability to
interact with the influenza A virus NP. The NPI-1 is
the human homolog of the yeast protein SRP1.
Interaction of NPI-1 and NP was demonstrated in yeast
5 by the interactive trap system; *in vitro*
coprecipitation of the NP with a bacterially expressed
NPI-1 protein; and in infected cell extracts by
coprecipitation of the NP with NPI-1, using anti-NPI-1
sera. The demonstration of this previously unknown
10 interaction is illustrated in the working examples
(see Section 6, *infra*). The data generated indicate
that NPI-1 plays a role in the replication of
influenza A viruses. NPI-1 is the first cellular
protein characterized which interacts with a protein
15 encoded by influenza viruses. This role, therefore,
makes the inhibition of the NP-NPI-1 interaction an
excellent target for antiviral therapy. It has not
yet been demonstrated at what stage in the replication
cycle NPI-1 functions. The NPI-1 could affect any of
20 a number of NP functions which may include: (1)
movement of the ribonucleoprotein complex (RNP) to the
nucleus during viral entry; (2) vRNA synthesis,
including antitermination and elongation; (3) mRNA
synthesis, including elongation, polyadenylation, and
25 transport to the cytoplasm; and (4) exit of the RNP
from the nucleus during virion assembly.

The fact that both NPI-1 and SRP1 interact with
proteins involved in RNA synthesis implies that there
may be fundamental similarities between cellular
30 DNA-dependent transcription and influenza viral
RNA-dependent RNA synthesis. Cellular factors, like
NPI-1, may be shared by the viral and the cellular RNA
synthesis machinery to perform similar functions. In
addition, the NPI-1 may tether the viral RNP to areas
35 of the nuclear matrix where splicing and

polyadenylation of mRNA occur. It should be noted
that although NPI-1 was isolated from HeLa cells, this
cell line is not productively infected by influenza A
virus. However, HeLa cells synthesize influenza viral
RNAs and proteins (see Fig. 6, lane 3), and have
5 previously been used to examine viral RNA synthesis
(Beaton & Krug, 1986, *supra*).

The viral NP exists in two forms in the infected
cell. One form is associated with ribonucleoprotein
complexes (RNP), and the other is a free form
10 (Shapiro & Krug, 1988, *supra*). Pol/NP preparations
used in coprecipitation experiments with NPI-1 were
purified over cesium chloride/glycerol gradients
(Honda et al., 1988, *supra*), which dissociate and
purify virion proteins away from vRNA. The NP but not
15 the polymerase proteins were detected on Coomassie
stained gels in this experiment (Fig 4, lane 3);
however, coprecipitation of the viral polymerase
proteins was not rigorously tested by immunoblot
experiments. Only the NP was coprecipitated from
20 infected HeLa cell extracts (Fig. 6) suggesting that
it is free NP which is bound by NPI-1.

Only one host factor has been assigned a
definitive function in the replication process of a
negative strand RNA virus. The cellular casein kinase
25 II has been shown to phosphorylate the phosphoprotein
P of the vesicular stomatitis virus (VSV) RNA-
dependent RNA polymerase. This is a step which
appears to be required in order to activate the viral
polymerase (Barik and Banerjee, 1992, Proc. Natl.
30 Acad. Sci. USA 89: 6570-6574; Barik and Banerjee,
1992, J. Virol. 66: 1109-1118).

NPI-1 and SRP1 are 50% identical and 81%
conserved at the amino acid level. This is a very
high degree of conservation between proteins belonging
35 to organisms as distantly related as humans and yeast,

and suggests that the NPI-1/SRP1 performs a very basic
function in the cell. NPI-1 and SRP1 have eight
internal repeats, each of approximately 42-amino acids
(Fig. 3). This repeat, termed the ARM motif, was
originally identified in the *Drosophila* segment
5 polarity gene *armadillo* (Riggleman, et al., 1989,
Genes Dev. 3: 96-113), and it has been identified in a
number of other proteins including β-catenin,
plakoglobin, p120, APC and smGDS (Peifer et al., 1994,
supra, and references therein). Several ARM proteins
10 are associated with cell adhesion structures.
Armadillo and its homologues bind to the C-terminal
cytoplasmic tail of cadherins, a calcium-dependent
class of cell adhesion molecules(CAMs), linking the
CAMs to the underlying cytoskeleton at cell-cell
15 junctions (McCrea, et al., 1991, Science 254: 1359-
1361). In contrast to the *armadillo* protein, SRP1 and
NPI-1 appear to be localized to the nucleus. If
NPI-1, like SRP1 (Yano, et al., 1992, Mol. Cell. Biol.
12: 5640-5651), is associated with the nuclear
20 membrane, it is possible that NPI-1 functions to
tether viral RNP to the nuclear membranes (Jackson,
et al., 1982, Nature 296: 366-368). It should be
noted that NPI-1 may be related to (or identical with)
a nuclear protein that has been found to be involved
25 in V(D)J recombination (Cuomo et al., 1994, Meeting
abstract F015, Keystone Symposium on Recombination).

The carboxyl terminal 265 amino acids of the
NPI-1, which were sufficient for interaction with the
viral NP, contain four and one-half ARM repeats.
30 Individual repeats, in general, are approximately 30 %
identical with the ARM consensus sequence. This is
consistent with the degree of conservation in ARM
repeats of other proteins (Peifer et al., 1994,
supra).

35

Using the same interactive trap system in yeast,
five additional DNA sequences were isolated which
partially encode proteins that interact with the NP of
influenza A virus. Also, using this system, a DNA
sequence encoding the NS1I-1 protein was identified
5 based the interaction between NS1I-1 and the NS1
protein of influenza A virus. This protein is the
human homolog of porcine 17β-estradiol dehydrogenase.
Several proteins with a dehydrogenase function have
recently been shown to be involved in post-
10 transcriptional events of gene expression (Hentze,
1994, Trends Biochem. Sci. 19: 101-103). This
supports an important functional role for the NS1I-1
interaction during the viral life cycle. The various
proteins so identified are listed in Table I.
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TABLE I

INTERACTING HOST CELL PROTEINS

Host Cell Proteins	FIG.	Comments
NPI-1	FIG. 2	New protein sequence, homologous to SRP1 of Yeast
NPI-2	FIG. 7	Identical to sequences of hnRNP C proteins (Lahiri & Thomas, 1986, Nucl. Acids Res. 14: 4077-4094)
NPI-3	FIG. 8	New protein sequence
NPI-4	FIG. 9	New protein sequence
NPI-5	FIG. 10	New protein sequence
NPI-6	FIG. 11	New protein sequence
NS1I-1	FIG. 12.	New protein sequence, homologous to porcine 17β-estradiol dehydrogenase

Note: Recently performed searches of Genebank have revealed that subsequent to Applicants' identification of NPI-3, NPI-4, and NPI-5, these sequences were described by other groups and designated Rch1, PC4, and BAT1, respectively.

The coding sequence for NPI-2 is identical to sequences coding for the previously identified hnRNP C proteins (Lahiri & Thomas, 1986, *supra*). The NPI-3, NPI-4, NPI-5, and NPI-6 coding sequences were unknown prior their identification by Applicants. The NS1I-1 gene is also novel, as explained in detail in the example in Section 7, below.

The invention contemplates, in addition to the DNA sequences disclosed herein, 1) any DNA sequence that encodes the same amino acid sequence as encoded by the DNA sequences shown in Figures 2 and 7-12; 2) any DNA sequence that hybridizes to the complement of the coding sequences disclosed herein (see Figs. 2 and

7-12) under highly stringent conditions, e.g., washing
in 0.1xSSC/0.1% SDS at 68°C (Ausubel, F.M. et al.,
eds., 1989, Current Protocols in Molecular Biology,
Vol. I, Green Publishing Associates, Inc., and John
Wiley & sons, Inc., New York, at p. 2.10.3) and
5   encodes a functionally equivalent gene product; and/or
3) any DNA sequence that hybridizes to the complement
of the coding sequences disclosed herein (see Figs. 2
and 7-12) under less stringent conditions, such as
moderately stringent conditions, e.g., washing in
10 0.2xSSC/0.1% SDS at 42°C (Ausubel, et al., 1989,
supra), yet which still encodes a functionally
equivalent gene product.

The invention also encompasses 1) DNA vectors
that contain any of the coding sequences disclosed
15 herein (see Figs. 2 and 7-12), and/or their
complements (i.e., antisense); 2) DNA expression
vectors that contain any of the coding sequences
disclosed herein (see Figs. 2 and 7-12), and/or their
complements (i.e., antisense), operatively associated
20 with a regulatory element that directs the expression
of the coding and/or antisense sequences; and 3)
genetically engineered host cells that contain any of
the coding sequences disclosed herein (see Figs. 2 and
7-12), and/or their complements (i.e., antisense),
25 operatively associated with a regulatory element that
directs the expression of the coding and/or antisense
sequences in the host cell. Regulatory element
includes but is not limited to inducible and non-
inducible promoters, enhancers, operators and other
30 elements known to those skilled in the art that drive
and regulate expression. The invention includes
fragments of any of the DNA sequences disclosed
herein.

Once the host cell proteins are obtained, they
35 can be used to detect interactions with proteins from

other viruses, in accordance with the invention. The following description is provided to illustrate this approach and not by way of limitation. Influenza B virus ribonucleoprotein complex was isolated and using a Western immunoblot assay, it was found that the

5   cellular NPI-1 was associated with this complex. This result indicates that NPI-1, isolated based on its interaction with influenza A virus NP, also interacts with influenza B virus NP. Thus, compounds that inhibit NP-NPI-1 interactions in influenza A virus and

10   thereby inhibit influenza A viral infection should be similarly effective as antivirals against influenza B virus.

Host cell genes that are homologous to those identified herein may be obtained by several methods.

15   In some cases, different host cell proteins that share the property of interacting with the same viral protein, e.g. influenza A virus NP, may also share genetic homology. Thus, the genes identified through the interactive trap selection may be homologous to

20   one another.

Once a host cell gene is identified in accordance with the invention, any homologous gene may be obtained using cloning methods well known to those skilled in the art, including but not limited to the

25   use of appropriate probes to detect the homologous genes within an appropriate cDNA or gDNA (genomic DNA) library. (See, for example, Sambrook et al., 1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratories, which is incorporated by

30   reference herein its entirety. This method is especially useful for obtaining proteins that may not share the property of binding to the same viral protein, but may nonetheless be genetically homologous.

35

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Such homologous proteins may interact with
proteins of viruses other than the virus used in the
interactive trap. For example, a host cell gene whose
product was detected through its interaction with an
influenza A viral protein may be homologous to another
5 gene whose product does not interact with influenza A
virus, but which does interact with influenza B viral
protein. To optimize the detection of such a
homologous gene, cDNA libraries may be constructed
from cells infected with a virus of interest. Besides
10 influenza B virus, this procedure may be applied
analogously to other viruses as well, including but
not limited to paramyxoviruses, such as parainfluenza
viruses, measles viruses, respiratory syncytial virus,
bunyviruses, arena viruses, the orthomyxo-like insect
15 virus called Dhori, as well as human immunodeficiency
virus (HIV), members of the herpes virus family, and
adenoviruses.

            5.2. SCREENING ASSAYS FOR COMPOUNDS THAT
20   INTERFERE WITH THE INTERACTION OF
                  HOST CELL AND VIRAL PROTEINS
                  REQUIRED FOR VIRAL REPLICATION

            The host cell protein and the viral protein which
interact and bind are sometimes referred to herein as
"binding partners". This term also includes peptide
25 fragments, produced as described in the subsections
below, comprising the binding domain of each
respective protein. Any of a number of assay systems
may be utilized to test compounds for their ability to
interfere with the interaction of the binding
30 partners. However, rapid high throughput assays for
screening large numbers of compounds, including but
not limited to ligands (natural or synthetic),
peptides, or small organic molecules are preferred.
·Compounds that are so identified to interfere with the
35 interaction of the binding partners should be further

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evaluated for antiviral activity in cell based assays,
animal model systems and in patients as described
herein.

The basic principle of the assay systems used to
identify compounds that interfere with the interaction
5 between the viral and host cell proteins involves
preparing a reaction mixture containing the viral
protein and the host cell protein under conditions and
for a time sufficient to allow the two proteins to
interact and bind, thus forming a complex. In order
10 to test a compound for inhibitory activity, the
reaction is conducted in the presence and absence of
the test compound, i.e., the test compound may be
initially included in the reaction mixture, or added
at a time subsequent to the addition of the viral and
15 host cell protein; controls are incubated without the
test compound or with a placebo. The formation of any
complexes between the viral protein and the host cell
protein is then detected. The formation of a complex
in the control reaction, but not in the reaction
20 mixture containing the test compound indicates that
the compound interferes with the interaction of the
viral protein and host cell protein.

The assay components and various formats that may
be utilized are described in the subsections below.
25

5.2.1. ASSAY COMPONENTS

The host cell protein and viral protein binding
partners used as components in the assay may be
derived from natural sources, e.g., purified from
30 cells and virus, respectively, using protein
separation techniques well known in the art; produced
by recombinant DNA technology using techniques known
in the art (see e.g., Sambrook et al., 1989, Molecular
Cloning: A Laboratory Manual, Cold Spring Harbor
35 Laboratories Press, Cold Spring Harbor, N.Y.); and/or

chemically synthesized in whole or in part using
techniques known in the art; e.g., peptides can be
synthesized by solid phase techniques, cleaved from
the resin and purified by preparative high performance
liquid chromatography (see, e.g., Creighton, 1983,
5   Proteins: Structures and Molecular Principles,
W.H. Freeman & Co., N.Y., pp. 50-60). The composition
of the synthetic peptides may be confirmed by amino
acid analysis or sequencing; e.g., using the Edman
degradation procedure (see e.g., Creighton, 1983,
10 supra at pp. 34-49).

The peptide fragments should be produced to
correspond to the binding domains of the respective
proteins. Any number of methods routinely practiced
in the art can be used to identify and isolate the
15 protein's binding site. These methods include but are
not limited to mutagenesis of one of the genes
encoding the protein and screening for disruption of
binding in a co-immunoprecipitation assay, or
mutagenesis of the host cell gene and selecting for
20 resistance to viral infection. Compensating mutations
in the viral gene can be selected which allow for
viral growth in this mutant host. Sequence analysis
of the genes encoding the respective proteins will
reveal the mutations that correspond to the region of
25 the protein involved in interactive binding.
Alternatively, one protein can be anchored to a solid
surface using methods described in section 5.2.2.
infra, and allowed to interact with and bind to its
labeled binding partner, which has been treated with a
30 proteolytic enzyme, such as trypsin. After washing, a
short, labeled peptide comprising the binding domain
may remain associated with the solid material, which
can be isolated and identified by amino acid
sequencing. Also, once the gene for the protein is
35 obtained, short gene segments can be engineered to

express peptide fragments of the protein, which can
then be tested for binding activity and purified or
synthesized.

Whether produced by molecular cloning methods or
by chemical synthetic methods, the amino acid sequence
5 of the binding partners which may be used in the
assays of the invention need not be identical to the
reported sequence of the genes encoding them. The
binding partners may comprise altered sequences in
which amino acid residues are deleted, added, or
10 substituted resulting in a functionally equivalent
product.

For example, functionally equivalent amino acid
residues may be substituted for residues within the
sequence resulting in a change of sequence. Such
15 substitutes may be selected from other members of the
class to which the amino acid belongs; e.g., the
nonpolar (hydrophobic) amino acids include alanine,
leucine, isoleucine, valine, proline, phenylalanine,
tryptophan, and methionine; the polar neutral amino
20 acids include glycine, serine, threonine, cysteine,
tyrosine, asparagine, and glutamine; the positively
charged (basic) amino acids include arginine, lysine,
and histidine; the negatively charged (acidic) amino
acids include aspartic and glutamic acid.

25   One of the binding partners used in the assay
system should be labeled, either directly or
indirectly, to facilitate detection of a complex
formed between the viral and host cell proteins. Any
of a variety of suitable labeling systems may be used
30 including but not limited to radioisotopes such as ^{125}I;
enzyme labelling systems that generate a detectable
colorimetric signal or light when exposed to
substrate; and fluorescent labels.

Where recombinant DNA technology is used to
35 produce the viral and host cell binding partners of

the assay it may be advantageous to engineer fusion
proteins that can facilitate labeling, immobilization
and/or detection. For example, the coding sequence of
the viral or host cell protein can be fused to that of
a heterologous protein that has enzyme activity or

5   serves as an enzyme substrate in order to facilitate
labeling and detection. The fusion constructs should
be designed so that the heterologous component of the
fusion product does not interfere with binding of the
host cell and viral protein.

10   Indirect labeling involves the use of a third
protein, such as a labeled antibody, which
specifically binds to one of the binding partners,
i.e., either the host cell protein or viral protein
used. Such antibodies include but are not limited to

15   polyclonal, monoclonal, chimeric, single chain, Fab
fragments and fragments produced by an Fab expression
library.

         For the production of antibodies, various host
animals may be immunized by injection with the host

20   cell protein or the viral protein, or a portion
thereof. Such host animals may include but are not
limited to rabbits, mice, and rats, to name but a few.
Various adjuvants may be used to increase the
immunological response, depending on the host species,

25   including but not limited to Freund's (complete and
incomplete), mineral gels such as aluminum hydroxide,
surface active substances such as lysolecithin,
pluronic polyols, polyanions, peptides, oil emulsions,
keyhole limpet hemocyanin, dinitrophenol, and

30   potentially useful human adjuvants such as BCG
(bacille Calmette-Guerin) and *Corynebacterium parvum*.

         Monoclonal antibodies may be prepared by using
any technique which provides for the production of
antibody molecules by continuous cell lines in

35   culture. These include but are not limited to the

hybridoma technique originally described by Kohler and
Milstein, (Nature, 1975, 256:495-497), the human
B-cell hybridoma technique (Kosbor et al., 1983,
Immunology Today, 4:72, Cote et al., 1983, Proc. Natl.
Acad. Sci., 80:2026-2030) and the EBV-hybridoma
5   technique (Cole et al., 1985, Monoclonal Antibodies
and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96).
In addition, techniques developed for the production
of "chimeric antibodies" (Morrison et al., 1984, Proc.
Natl. Acad. Sci., 81:6851-6855; Neuberger et al.,
10 1984, Nature, 312:604-608; Takeda et al., 1985,
Nature, 314:452-454) by splicing the genes from a
mouse antibody molecule of appropriate antigen
specificity together with genes from a human antibody
molecule of appropriate biological activity can be
15 used. Alternatively, techniques described for the
production of single chain antibodies (U.S. Patent
No. 4,946,778) can be adapted to produce single chain
antibodies specific to one of the binding partners.
        Antibody fragments which recognize specific
20 epitopes may be generated by known techniques. For
example, such fragments include but are not limited
to: the $F(ab')_2$ fragments which can be produced by
pepsin digestion of the antibody molecule and the Fab
fragments which can be generated by reducing the
25 disulfide bridges of the $F(ab')_2$ fragments.
Alternatively, Fab expression libraries may be
constructed (Huse et al., 1989, Science, 246:1275-
1281) to allow rapid and easy identification of
monoclonal Fab fragments with the desired specificity.
30

5.2.2. ASSAY FORMATS
        The assay can be conducted in a heterogeneous or
homogeneous format. Heterogeneous assays involve
anchoring one of the binding partners onto a solid
35 phase and detecting complexes anchored on the solid

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phase at the end of the reaction. In homogeneous
assays, the entire reaction is carried out in a liquid
phase. In either approach, the order of addition of
reactants can be varied to obtain different
information about the compounds being tested. For
5   example, test compounds that interfere with the
interaction between the binding partners, e.g., by
competition, can be identified by conducting the
reaction in the presence of the test substance; i.e.,
by adding the test substance to the reaction mixture
10 prior to or simultaneously with the viral protein and
host cell protein. On the other hand, test compounds
that disrupt preformed complexes, e.g. compounds with
higher binding constants that displace one of the
binding partners from the complex, can be tested by
15 adding the test compound to the reaction mixture after
complexes have been formed. The various formats are
described briefly below.

In a heterogeneous assay system, one binding
partner, e.g., either the viral protein or the host
20 cell protein, is anchored onto a solid surface, and
its binding partner, which is not anchored, is
labeled, either directly or indirectly. In practice,
microtiter plates are conveniently utilized. The
anchored species may be immobilized by non-covalent or
25 covalent attachments. Non-covalent attachment may be
accomplished simply by coating the solid surface with
a solution of the protein and drying. Alternatively,
an immobilized antibody specific for the protein may
be used to anchor the protein to the solid surface.
30 The surfaces may be prepared in advance and stored.

In order to conduct the assay, the binding
partner of the immobilized species is added to the
coated surface with or without the test compound.
After the reaction is complete, unreacted components
35 are removed (e.g., by washing) and any complexes

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formed will remain immobilized on the solid surface.
The detection of complexes anchored on the solid
surface can be accomplished in a number of ways.
Where the binding partner was pre-labeled, the
detection of label immobilized on the surface

5   indicates that complexes were formed. Where the
binding partner is not pre-labeled, an indirect label
can be used to detect complexes anchored on the
surface; e.g., using a labeled antibody specific for
the binding partner (the antibody, in turn, may be

10 directly labeled or indirectly labeled with a labeled
anti-Ig antibody). Depending upon the order of
addition of reaction components, test compounds which
inhibit complex formation or which disrupt preformed
complexes can be detected.

15   Alternatively, the reaction can be conducted in a
liquid phase in the presence or absence of the test
compound, the reaction products separated from
unreacted components, and complexes detected; e.g.,
using an immobilized antibody specific for one binding

20 partner to anchor any complexes formed in solution,
and a labeled antibody specific for the other binding
partner to detect anchored complexes. Again,
depending upon the order of addition of reactants to
the liquid phase, test compounds which inhibit complex

25 or which disrupt preformed complexes can be
identified.

        In an alternate embodiment of the invention, a
homogeneous assay can be used. In this approach, a
preformed complex of the host cell and viral protein

30 is prepared in which one of the binding partners is
labeled, but the signal generated by the label is
quenched due to complex formation (see, e.g., U.S.
Patent No. 4,109,496 by Rubenstein which utilizes this
approach for immunoassays). The addition of a test

35 substance that competes with and displaces one of the

binding partners from the preformed complex will
result in the generation of a signal above background.
In this way, test substances which disrupt the viral
protein-host cell protein interaction can be
identified.

5   For example, in a particular embodiment for
NPI-1, NPI-1 can be prepared for immobilization using
recombinant DNA techniques described in section
5.2.1., *supra*. Its coding region can be fused to the
glutathione-S-transferase (GST) gene using the fusion
10   vector pGEX-5X-1, in such a manner that its binding
activity is maintained in the resulting fusion
protein. NP can be purified and used to raise a
monoclonal antibody, specific for NP, using methods
routinely practiced in the art and described above.
15   This antibody can be labeled with the radioactive
isotope ^{125}I, for example, by methods routinely
practiced in the art. In a heterogeneous assay, e.g.,
the GST-NPI-1 fusion protein can be anchored to
glutathione-agarose beads. NP can then be added in
20   the presence or absence of the test compound in a
manner that allows NP to interact with and bind to the
NPI-1 portion of the fusion protein. After the test
compound is added, unbound material can be washed
away, and the NP-specific labeled monoclonal antibody
25   can be added to the system and allowed to bind to the
complexed binding partners. The interaction between
NP and NPI-1 can be detected by measuring the amount
of radioactivity that remains associated with the
glutathione-agarose beads. A successful inhibition of
30   the interaction by the test compound will result in a
decrease in measured radioactivity.

        Alternatively, the GST-NPI-1 fusion protein and
NP can be mixed together in liquid in the absence of
the solid glutathione-agarose beads. The test
35   compound can be added either during or after the

binding partners are allowed to interact. This
mixture can then be added to the glutathione-agarose
beads and unbound material is washed away. Again the
extent of inhibition of the binding partner
interaction can be detected by measuring the
5 radioactivity associated with the beads.

In another embodiment of the invention, these
same techniques can be employed using peptide
fragments that correspond to the binding domains of NP
and NPI-1, respectively, in place of one or both of
10 the full length proteins. These binding domains can
be identified, as described in section 5.2.1., *supra*.
For example, and not by way of limitation, NPI-1 can
be anchored to a solid material as described above in
this section by making a GST-NPI-1 fusion protein and
15 allowing it to bind to glutathione agarose beads. NP
can be labeled with a radioactive isotope, such as ^{35}S,
and cleaved with a proteolytic enzyme such as trypsin.
Cleavage products can then be added to the anchored
GST-NPI-1 fusion protein and allowed to bind. After
20 washing away unbound peptides, labeled bound material,
representing the NP binding domain, can be eluted,
purified, and analyzed for amino acid sequence by
methods described in section 5.2.1., *supra*. Peptides
so identified can be produced synthetically or fused
25 to appropriate facilitative proteins using recombinant
DNA technology, as described in section 5.2.1., *supra*.

In accordance with the invention, a given
compound found to inhibit one virus may be tested for
general antiviral activity against a wide range of
30 different viruses that have analogous dependencies on
host cell proteins. For example, and not by way of
limitation, a compound which inhibits the interaction
of influenza virus NP with NPI-1 by binding to the NP
binding site can be tested, according to the assays
35 described in section 5.3. *infra*, against other

viruses, particularly those which have similar proteins, _e.g._, parainfluenza viruses.

5.3.   ASSAYS FOR ANTIVIRAL ACTIVITY

Any of the inhibitory compounds which are
identified in the foregoing assay systems may be
tested for antiviral activity.

5.3.1.   VIRAL GROWTH ASSAYS

The ability of an inhibitor identified in the
foregoing assay systems to prevent viral growth can be
assayed by plaque formation or by other indices of
viral growth, such as the $TCID_{50}$ or growth in the
allantois of the chick embryo. In these assays, an
appropriate cell line or embryonated eggs are infected
with wild-type influenza virus, and the test compound
is added to the tissue culture medium either at or
after the time of infection. The effect of the test
compound is scored by quantitation of viral particle
formation as indicated by hemagglutinin (HA) titers
measured in the supernatants of infected cells or in
the allantoic fluids of infected embryonated eggs; by
the presence of viral plaques; or, in cases where a
plaque phenotype is not present, by an index such as
the $TCID_{50}$ or growth in the allantois of the chick
embryo, or with a hemagglutination assay.

An inhibitor can be scored by the ability of a
test compound to depress the HA titer or plaque
formation, or to reduce the cytopathic effect in
virus-infected cells or the allantois of the chick
embryo, or by its ability to reduce viral particle
formation as measured in a hemagglutination assay.

5.3.2 ANIMAL MODEL ASSAYS

The ability of an inhibitor to prevent
replication of influenza virus can be assayed in

animal models that are natural or adapted hosts for
influenza. Such animals may include mammals such as
pigs, ferrets, mice, monkeys, horses, and primates, or
birds. As described in detail in Section 5.5 *infra*,
such animal models can be used to determine the LD_{50}
5   and the ED_{50} in animal subjects, and such data can be
used to derive the therapeutic index for the inhibitor
of the viral/host cell protein interaction.

5.4. INHIBITORY COMPOUNDS

10   Inhibitory compounds identified in the foregoing
screening assays which may be used in accordance with
the invention may include but are not limited to small
organic molecules, peptides and antibodies.

      For example, peptides having an amino acid
15   sequence corresponding to the domain of the host cell
protein that binds to the viral protein may be used to
compete with the native viral protein and, therefore,
may be useful as inhibitors in accordance with the
invention. Similarly, peptides having an amino acid
20   sequence corresponding to the domain of the viral
protein that binds to the host cell protein may be
used. Such peptides may be synthesized chemically or
produced via recombinant DNA technology using methods
well known in the art (e.g., see Creighton, 1983,
25   *supra*; and Sambrook et al., 1989, *supra*). Lipofectin
or liposomes may be used to deliver the peptides to
cells.

      Alternatively, antibodies that are both specific
for the binding domains of either the host cell or
30   viral proteins and interfere with their interaction
may be used. Such antibodies may be generated using
standard techniques described in Section 5.2.1.,
supra, against the proteins themselves or against
peptides corresponding to the binding domains of the
35   proteins. Such antibodies include but are not limited

to polyclonal, monoclonal, Fab fragments, single chain antibodies, chimeric antibodies, etc. Where whole antibodies are used, internalizing antibodies are preferred. However, lipofectin may be used to deliver the antibody or a fragment of the Fab region which

5   binds to the viral or host cell protein epitope into cells. Where fragments of the antibody are used, the smallest inhibitory fragment which binds to the target protein's binding domain is preferred.

10   ### 5.5. PHARMACEUTICAL PREPARATIONS AND METHODS OF ADMINISTRATION

The identified compounds that inhibit viral replication can be administered to a patient at therapeutically effective doses to treat viral

15   infection. A therapeutically effective dose refers to that amount of the compound sufficient to result in amelioration of symptoms of viral infection.

Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical

20   procedures in cell cultures or experimental animals, e.g., for determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects

25   is the therapeutic index and it can be expressed as the ratio LD_{50}/ED_{50}. Compounds which exhibit large therapeutic indices are preferred. While compounds that exhibit toxic side effects may be used, care should be taken to design a delivery system that

30   targets such compounds to the site of infection in order to minimize damage to uninfected cells and reduce side effects.

The data obtained from the cell culture assays and animal studies can be used in formulating a range

35   of dosage for use in humans. The dosage of such

compounds lies preferably within a range of
circulating concentrations that include the ED50 with
little or no toxicity. The dosage may vary within
this range depending upon the dosage form employed and
the route of administration utilized. For any
5 compound used in the method of the invention, the
therapeutically effective dose can be estimated
initially from cell culture assays. A dose may be
formulated in animal models to achieve a circulating
plasma concentration range that includes the IC50
10 (_i.e._, the concentration of the test compound which
achieves a half-maximal infection, or a half-maximal
inhibition) as determined in cell culture. Such
information can be used to more accurately determine
useful doses in humans. Levels in plasma may be
15 measured, for example, by high performance liquid
chromatography.

Pharmaceutical compositions for use in accordance
with the present invention may be formulated in
conventional manner using one or more physiologically
20 acceptable carriers or excipients.

Thus, the compounds and their physiologically
acceptable salts and solvates may be formulated for
administration by inhalation or insufflation (either
through the mouth or the nose) or oral, buccal,
25 parenteral or rectal administration.

For administration by inhalation, the compounds
for use according to the present invention are
conveniently delivered in the form of an aerosol spray
presentation from pressurized packs or a nebuliser,
30 with the use of a suitable propellant, _e.g._,
dichlorodifluoromethane, trichlorofluoromethane,
dichlorotetrafluoroethane, carbon dioxide or other
suitable gas. In the case of a pressurized aerosol
the dosage unit may be determined by providing a valve
35 to deliver a metered amount. Capsules and cartridges

of e.g. gelatin for use in an inhaler or insufflator
may be formulated containing a powder mix of the
compound and a suitable powder base such as lactose or
starch.

For oral administration, the pharmaceutical
5   compositions may take the form of, for example,
tablets or capsules prepared by conventional means
with pharmaceutically acceptable excipients such as
binding agents (e.g., pregelatinised maize starch,
polyvinylpyrrolidone or hydroxypropyl
10 methylcellulose); fillers (e.g., lactose,
microcrystalline cellulose or calcium hydrogen
phosphate); lubricants (e.g., magnesium stearate, talc
or silica); disintegrants (e.g., potato starch or
sodium starch glycollate); or wetting agents (e.g.,
15 sodium lauryl sulphate). The tablets may be coated by
methods well known in the art. Liquid preparations
for oral administration may take the form of, for
example, solutions, syrups or suspensions, or they may
be presented as a dry product for constitution with
20 water or other suitable vehicle before use. Such
liquid preparations may be prepared by conventional
means with pharmaceutically acceptable additives such
as suspending agents (e.g., sorbitol syrup, cellulose
derivatives or hydrogenated edible fats); emulsifying
25 agents (e.g., lecithin or acacia); non-aqueous
vehicles (e.g., almond oil, oily esters, ethyl alcohol
or fractionated vegetable oils); and preservatives
(e.g., methyl or propyl-p-hydroxybenzoates or sorbic
acid). The preparations may also contain buffer
30 salts, flavoring, coloring and sweetening agents as
appropriate.

Preparations for oral administration may be
suitably formulated to give controlled release of the
active compound.
35

For buccal administration the compositions may take the form of tablets or lozenges formulated in conventional manner.

The compounds may be formulated for parenteral administration by injection, e.g., by bolus injection

5 or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous

10 vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use.

15   The compounds may also be formulated in rectal compositions such as suppositories or retention enemas, e.g., containing conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described

20 previously, the compounds may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds may be

25 formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

The compositions may, if desired, be presented in

30 a pack or dispenser device which may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions

35 for administration.

6. EXAMPLE: THE IDENTIFICATION OF NPI-1
AND ITS INTERACTION WITH
INFLUENZA NUCLEOPROTEIN

The yeast interactive trap system was used to
identify a cellular protein which interacts with the
nucleoprotein of influenza A viruses. This protein,
nucleoprotein interactor 1 (NPI-1) is the human
homologue of the yeast protein SRP1. SRP1 was
previously identified as a suppressor of temperature-
sensitive RNA polymerase I mutations (Yano, et. al.,
1992, Mol. Cell. Biol. 12:5640-5651). A full length
cDNA clone of NPI-1 was generated from HeLa cell poly
A+ RNA. The viral NP, which had been partially
purified from influenza A/PR/8/34 virus-infected
embryonated eggs, could be coprecipitated from
solution by glutathione agarose beads complexed with a
bacterially expressed glutathione-S-transferase (GST)-
NPI-1 fusion protein, confirming the results of the
yeast genetic system. Antisera raised against NPI-1
identified a 65 kDa polypeptide from total cellular
extracts of both HeLa and MDBK cells. In addition,
the viral nucleoprotein was co- immunoprecipitated
from influenza A/WSN/33 virus-infected HeLa cells by
antisera directed against NPI-1, demonstrating an
interaction of these two proteins in infected cells,
and suggesting that NPI-1 plays a role during
influenza virus replication.

6.1. MATERIALS AND METHODS

6.1.1. YEAST, BACTERIA AND PLASMIDS

Yeast strain EGY48 (Mata trp1 ura3 his3
LEU2::pLEXAop6-LEU2) (Zervos et al., 1993, Cell 72:
222-232) and plasmids pEG202, pSH18-34, and pRFHM1 and
the HeLa cell cDNA library constructed in pJG4-5
(Gyuris et al., 1993, Cell 75: 791-803) were

previously described. Similar versions of these
plasmids and this yeast host strain are available
commercially from Clontech as part of a two fusion
protein system. pLexA-NP was constructed by
subcloning the cDNA of influenza A/PR/8/34 NP as a
5 LexA translational fusion gene into pEG202 (Fig. 1).
Yeast strains constructed as part of these studies are
described in Table 2. *Escherichia coli* MH3 (*trpC araD
lacX hsdR galU galK*) and W31005 were previously
described (Hall et al., 1984, Cell 36: 1057-1065).
10

6.1.2.   SELECTION OF NP INTERACTORS

An interactive trap selection was performed
essentially as has been previously described (Gyuris,
et al., 1993, *supra*; Zervos, et al., 1993, *supra*).
15 Strain R100 was transformed by the HeLa cDNA library
using the lithium acetate method (Ito, et al., 1983,
J. Bacteriol. 153: 163-168). 2×10^6 primary yeast
transformants were selected on twelve 25×25 cm^2 his⁻
trp⁻-glucose plates, pooled and stored at -70°C.
20 Library transformants were selected for leu+ phenotype
on his⁻leu⁻-galactose plates; the efficiency of plating
was approximately 10^{-4} leu+ colonies per galactose+
colony. Plasmid DNA was isolated from leu+ library
transformants as described by Hoffman and Winston
25 (Hoffman & Winston, 1987, Gene 57: 267-272) and
introduced into MH3 cells by electroporation. Library
plasmids were selected by plating the transformation
mix on 1xA+amp+glucose plates (Miller, 1972,
Experiments in Molecular Genetics, Cold Spring Harbor
30 Laboratory Press, Cold Spring Harbor, NY).

cDNAs were analyzed by checking the specificity
of interaction with the NP. Each isolated plasmid was
introduced into strains R101 and R102. These strains
harbor pSH18-34, a reporter plasmid encoding
35

β-galactosidase with a GAL1 promoter transcriptionally
controlled from upstream LexA binding sites. Strain
R102 was used as a negative control for NP-specificity
of cloned cDNAs. It contains pRFHM1, which encodes
LexA fused to a transcriptionally inert fragment of
5 the *Drosophila melanogaster* bicoid protein.
β-Galactosidase activity was assayed on nitrocellulose
replicas of the colonies by freeze fracturing the
cells and incubating in buffer containing 5-bromo-4-
chloro-3-indolyl-β-D-galactoside (X-gal) (Miller,
10 1972, *supra*). Plasmids which conferred both a leu+
and β-gal+ phenotypes in the presence of pLexA-NP but
not in the presence of pRFHM1 were saved for further
study.

15 6.1.3. CLONING OF THE 5' TERMINUS OF NPI-1
The 5' terminus of NPI-1 was cloned by rapid
amplification of cDNA ends ("RACE") by the method of
Frohman (Frohman, 1990, in PCR Protocols: A Guide to
Methods and Applications, Innis et. al., eds.,
20 Academic Press Inc., San Diego, p. 28-38; Frohman,
et al., 1988, Proc. Natl. Acad. Sci. USA 85: 8998-
9002). Reverse transcription of 1 μg of poly A+ HeLa
cell RNA was performed using the NPI-1 specific
oligonucleotide 5'GCAAAGCAGGAGAAACCAC3'. First strand
25 cDNA was tailed with dCTP by terminal transferase.
PCR amplification of the reverse transcription product
was performed with the nested NPI-1 primer
5'GGGTCCATCTGATAGATATGAGAG3' and the 5' RACE anchor
primer
30 5'CUACUACUACUAGGCCACGCGTCGACTACTACGGGIIGGGIIGGGIIG3'
(Gibco/BRL). The PCR product was subcloned into
pGEM-T (Promega) and was sequenced by standard
protocols. 5'RACE products from three independent
·experiments were cloned and sequenced in order to
35 avoid errors introduced by PCR.

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6.1.4.   BACTERIAL EXPRESSION AND
PURIFICATION OF GST-NPI-1

The NPI-1 cDNA derived from a HeLa cDNA library
was subcloned into the *EcoR1* and *XhoI* restriction
endonuclease sites of the glutathione-S-transferase
5   fusion vector pGEX-5X-1 (Pharmacia) to generate the
plasmid pGST-NPI-1. Protein was induced from
bacterial expression plasmids in W31005 cells with
isopropyl-β-D-galactopyranoside according to standard
protocols (Smith & Johnson, 1988, Gene 67: 31-40).
10 Bacteria were pelleted 4 h after induction, washed in
ice cold phosphate buffered saline (PBS), and
resuspended in one-tenth culture volume PBS+1% Triton
X-100. Bacteria were lysed on ice with four 15 s
pulses in a Raytheon sonicator at an output setting of
15 1 amp. Insoluble material was pelleted at 50,000xg
for 30 min in a Beckman TL-100.3 rotor.

GST-NPI-1 and GST were purified from bacterial
lysates on glutathione-agarose beads(Sigma Chemical
Corp.). Beads were swelled according to the
20 manufacturer's instructions and equilibrated in PBS.
Typical binding reactions were done in 500 μl of
PBS/0.1% Triton X-100, and included 50 μl bacterial
lysate and 10 μl of a 50% slurry of glutathione-
agarose beads. Binding reactions were incubated for
25 5 min at room temperature on a rotating wheel. Beads
were collected by centrifugation for 5 s in a
microfuge, and were washed three times in PBS.

6.1.5.   NP BINDING ASSAY

30   To assay binding of NP to GST-NPI-1/bead
complexes typical reactions were performed in 500 μl
of ice cold PBS+0.05% Nonidet P-40 and contained
washed GST-NP1-1/bead complexes and 10 μg partially
purified influenza virus polymerase and nucleoprotein
35 preparations (Pol/NP). Virus was prepared from

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embryonated eggs infected by influenza A/PR/8/34 virus
and POL/NP preparations were purified as previously
described (Enami, et al., 1990, Proc. Natl. Acad. Sci.
USA 87: 3802-3805; Parvin, et al., 1989, J. Virol. 63:
5142-5152). NP was bound for 1 h at 4°C on a rotating
5   wheel. Beads were collected by centrifugation for 5 s
in a microfuge, and were washed three times in
PBS+0.05% NP-40. Washed beads were resuspended in
50 μl SDS sample buffer (Sambrook, et al., 1989,
Molecular Cloning: A Laboratory Manual, Cold Spring
10 Harbor Laboratories Press, Cold Spring Harbor, NY),
boiled for 5 min, and pelleted in a microfuge. 10 μl
of each supernatant was separated by electrophoresis
on a 12.5% SDS-polyacrylamide gel. Gels were either
stained with Coomassie blue or processed for
15 immunoblot analysis. NP was detected by
immunoblotting with the monoclonal antibody HT103.

6.1.6. ANTISERA AND IMMUNOBLOTTING

Polyclonal rabbit antisera against NPI-1 was
20 generated by immunization of a female NZY Rabbit
(Buckshire Farms) with 200 μg of purified GST-NPI-1 in
complete Freund's adjuvant, followed by two boosts of
100 μg in incomplete Freund's adjuvant at three week
intervals. The specificity of antisera was
25 demonstrated by immunoblot analysis of GST-NPI-1 in
bacterial lysates. Immunoblots were performed by
standard methods (Harlow and Lane, 1998, Antibodies: A
Laboratory Manual, Cold Spring Harbor Laboratories
Press, Cold Spring Harbor, NY). Sera were used at a
30 dilution of 1:1000.

6.1.7. VIRUSES AND CELLS

Total cell lysates from HeLa and MDBK cells were
generated by direct lysing of cells in SDS-sample
35 buffer, followed by shearing of chromosomal DNA by

passage through a 21 ga. syringe. Cytoplasmic
extracts were generated by lysing cells in ice cold
NP-40 lysis buffer (10 mM Tris-Cl, pH 8.0; 100 mM
NaCl; 1 mM EDTA; 1 mM DTT; 1% Nonidet P-40; 1 mM 4-(2-
aminoethyl)benzenesulfonylfluoride-hydrochloride
5 (Pefabloc)). After 10 min on ice nuclei were removed
by centrifugation. Proteins were separated by SDS-
PAGE, transferred to nitrocellulose and visualized by
immunoblotting.

To generate infected cell lysates containing
10 metabolically labeled proteins 4x10^6 HeLa cells were
infected with influenza A/WSN/33 virus at a
multiplicity of 10 for 45 min at 37°C. Infection was
allowed to proceed in DMEM + 0.1% BSA for 5 h at which
time cells were labeled with 50 μCi ^{35}S-methionine +
15 50 μCi ^{35}S-cystine in MEM-cys-met for 1 h. Extracts
were prepared by resuspending infected cells in 650 μl
ice cold NP-40 lysis buffer followed by two 15 s
pulses in a Raytheon sonicator to disrupt nuclei.
Insoluble cell debris was removed by centrifugation at
20 100,000xg in a TL-100.3 Beckman rotor. 5 μl anti-NPI-
1 sera was incubated on ice for 1 h with 100 μl
infected cell lysates. Immune complexes were
precipitated from solution by incubation with
Sepharose-4B linked protein G beads (Sigma) for 1 h.
25 Beads were collected by centrifugation, washed three
times in NP-40 lysis buffer, and resuspended in SDS-
sample buffer. Precipitated proteins were separated
by SDS-PAGE and visualized by autoradiography.

30   6.2. <u>RESULTS</u>

                 6.2.1. <u>ISOLATION OF NPI-1</u>
The interactive trap was used to identify
proteins which specifically interact with the
35

influenza A virus nucleoprotein (NP). The interactive
trap is one of several genetic systems recently
developed which uses the modular nature of
transcription activators to detect protein:protein
interactions (Chien, et al., 1991, Proc. Natl. Acad.
5 Sci. USA 88: 9578-9582; Dalton & Treisman, 1992, Cell
68: 597-612; Durfee, et al., 1993, Genes Dev. 7: 555-
569; Gyuris, et al., 1993, *supra*; Vojtek, et al.,
1993, Cell 74: 205-214; Zervos, et al., 1993, *supra*).
The interactive trap consists of three components:
10 (1) a reporter gene that has no basal transcription;
(2) a fusion protein which contains a LexA DNA binding
domain that is transcriptionally inert; and
(3) proteins encoded by an expression library, which
are expressed as fusion proteins containing an
15 activation domain (Fig. 1A). Interaction of the LexA
fusion protein and the fusion protein containing the
activation domain will constitute a bimolecular
transcriptional activator which, in this case, will
confer the ability to grow on media lacking leucine
20 (Gyuris, et al., 1993, *supra*; Zervos, et al., 1993,
supra). In the absence of this interaction the leu2
gene is not transcribed.

The NP gene of influenza A/PR/8/34 virus was
subcloned as a translational fusion gene with the LexA
25 gene into pEG202 to generate pLexA-NP (Fig. 1B).
Strain R100 (Table II), which contains pLexA-NP, was
transformed with a HeLa cell cDNA library constructed
in pJG4-5. pJG4-5 contains an activation domain under
control of a GAL1 promoter (Gyuris, et al., 1993,
30 *supra*).

TABLE II
YEAST STRAINS USED

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35 Strains   Genotype

EGY48	*Mata trp1 ura3 his3 LEU2::pLEXAop6-LEU2*
R100	EGY48, pLexA-NP(TRP1)
R101	EGY48, pLexA-NP, pSH18-34(HIS3)
R102	EGY48, pRFHM1(TRP1), pSH18-34

Library plasmids were rescued from 100 leu+ colonies.
Reproducibility of the interaction of the NP with the
encoded library proteins was tested by transforming
library plasmids into strain R101. Transformants were
screened for galactose-dependent β-galactosidase
activity and growth on media lacking leucine.
Specificity for NP was analyzed by checking the
ability of library plasmids to confer growth on leu⁻
media and β-galactosidase activity in connection with
a different LexA fusion plasmid, pRFHM1, encoding a
fragment of the *Drosophila melanogaster* bicoid
protein. Twenty-three library plasmids were confirmed
to encode NP-interactive proteins. Twelve identical
2.1 kbp clones encoded the carboxy terminal fragment
of a protein termed nucleoprotein interactor 1
(NPI-1). Partial DNA sequencing showed that NPI-1 is
the human homologue of the yeast SRP1 gene (*infra*).

6.2.2.   CLONING AND SEQUENCING OF THE NPI-1 cDNA

The 2.1 kbp NPI-1 cDNA in pJG4-5 was sequenced by
standard protocols. The 5' cDNA terminus of the NPI-1
gene was cloned by 5' RACE. cDNAs from 3
independently derived NPI-1 5'RACE products were
cloned and sequenced. Nucleotide and derived amino
acid sequences of NPI-1 are shown in Figure 2. The
sequence reveals a 2.9 kbp cDNA which encodes a
protein of 527 amino acids with a calculated molecular
weight of 58,754 Da and a pI = 4.74. The carboxyl

terminal 265 amino acids were encoded by the
interactive trap library plasmid and interact with the
viral NP.

Comparison of the deduced amino acid sequences in
the GenBank and EMBL data bases using the FASTA and
5 TFASTA programs (Deveraux, et al., 1984, Nucleic Acids
Res. 12: 387-395) demonstrated that NPI-1 is the human
homologue of the *Saccharomyces cerevisiae* protein SRP1
(Yano, et al., 1992, Mol. and Cell. Biol. 12: 5640-
5651). SRP1 was cloned as an allele-specific
10 suppressor of ts mutations in the zinc-binding domain
of the A190 subunit of RNA polymerase I. The amino
acid sequence is highly conserved between NPI-1 and
SRP1: 50% identity and 81% similarity at the amino
acid level. The amino terminus of NPI-1 has a
15 potential nuclear localization signal (Chelsky,
et al., 1989, Mol. Cell. Biol. 9:2487-2492); amino
acids 25 to 49 are rich in arginine, and contain a
stretch of four consecutive arginines at amino acids
28 to 31. NPI-1, like SRP1, contains a series of 8
20 consecutive ARM motifs, which are 42 amino acid
protein subsequences originally identified in the
Drosophila armadillo protein (Peifer et al., Cell 76:
789-791, 1994; Yano, et al., 1992, *supra*) (Fig. 3,
infra).
25

6.2.3. NPI-1 BINDS TO NP IN VITRO

In order to demonstrate that the NPI-1 binds to
the viral NP, the NPI-1 cDNA fragment (amino acids 262
to 527) was subcloned into the bacterial expression
30 vector pGEX-5X-1 yielding a glutathione S-transferase
fusion gene. The expressed fusion protein was
purified from bacterial lysates on glutathione agarose
beads. NP, which had been partially purified with the
viral polymerase from influenza A/PR/8/34 virus was
35 specifically precipitated from solution by glutathione

agarose beads complexed with GST-NPI-1 (Fig. 4). The
NP band migrates slightly faster than that of the GST-
NPI-1 fusion protein. The identity of this protein
was confirmed by immunoblot analysis using the anti-NP
monoclonal antibody HT103 (Fig 4, lane 8).

5

6.2.4. IMMUNODETECTION OF NPI-1 IN CELL EXTRACTS

Rabbit antisera raised against GST-NPI-1 were
used to identify a polypeptide from total cellular
extracts of both HeLa and MDBK cells with an apparent

10 molecular weight of 65 kDa (Fig. 5). The molecular
weight predicted from the derived amino acid sequence
of the cDNA is slightly smaller (59 kDa). A lower
amount of NPI-1 was present in the cytoplasmic
fraction generated by lysis of cells in the presence

15 of NP-40 than in the total cellular extract suggesting
that most of NPI-1 is located in the nucleus (Fig. 5).
This is consistent with results localizing the NPI-1
homologue SRP1 to the nucleus of yeast cells by
immunofluorescence (Yano, et al., 1992, *supra*).

20 Localization of NPI-1 to a particular intracellular
compartment by immunofluorescence experiments has not
been posssible due to the high background fluorescence
of the antisera preparations used.

25 ### 6.2.5. NPI-1 INTERACTS WITH NP IN INFECTED CELLS

Since NP formed a complex with NPI-1 *in vitro*, we
examined whether NP and NPI-1 form a complex in
infected cells. NP was specifically
coimmunoprecipitated from extracts of influenza A/WSN

30 virus infected HeLa cells by antisera directed against
NPI-1 (Fig 6). This demonstrates an interaction of
the viral NP and the cellular NPI-1 during influenza A
virus infection.

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7.   Example: THE IDENTIFICATION OF NS1I-1 AND ITS
                 INTERACTION WITH INFLUENZA
                 NUCLEOPROTEIN NS1

         In the example described below, the yeast
interactive trap system was used to identify a human
protein, NS1I-1 (NS1-interactor-1), from a HeLa cell

5   cDNA library on the basis of its binding to NS1 of
influenza A virus. NS1I-1 is shown herein to be
recognized not only by NS1 proteins from five human
and avian influenza A strains, but also by NS1 of
influenza B virus. Surprisingly, NS1I-1 is homologous

10   to a steroid dehydrogenase isolated from pigs
(Leenders, et al., 1994, Eur. J. Biochem. 222: 221-
227). Several proteins with a dehydrogenase function
have recently been shown not only to have enzymatic
activity but also to be involved in post-

15   transcriptional events of gene-expression (Hentze,
1994, *supra*). This strong conservation supports an
important functional role of the NS1I-1 interaction
during the viral life cycle.

20

                      7.1.   MATERIALS AND
METHODS

     7.1.1.   <u>YEAST, E.COLI STRAINS, AND PLASMIDS</u>
         Manipulations of nucleic acids, *Escherichia*

25   *coli* and yeast followed essentially standard
procedures as described elsewhere (Ausubel, et al.,
1992, Current Protocols in Molecular Biology, Green
Publishing Associates, Inc., and John Wiley & sons,
Inc., New York). The yeast strains EGY40 (*Mata trp1*

30   *ura3 his3*) and EGY48 (*Mata trp1 ura3 his3 LEU2::pLEX-*
Aop6-LEU2) as well as plasmids pEG202, pRFHM1, and
pSH18-34, and the HeLa cell cDNA constructed in pJG4-5
have been described (Gyuris, et al., 1993, *supra*;
Zervos, et al., 1993, *supra*). *E. coli* strains used

35

for cloning and expression were MH3 (trpC araD lacX
hsdR galU galK), DH5α (F⁻Φ80dlacZΔM15 Δ(lacZY-argF)U169
deoR recA1 endA1 hsdR17(r$_K$⁻m$_K$+) supE44λ⁻thi⁻ gyrA96
relA1), and BL26 (F⁻ompT hsdS$_B$(r$_B$⁻m$_B$⁻) gal dcm). pLexA-
NS1 was constructed by subcloning the cDNA of the NS

5 segment of influenza virus A/PR/8/34 downstream of the
LexA gene in pEG202. pGEX-NS1I-1 was constructed by
subcloning the HeLa cDNA-insert of library plasmid pK5
as an EcoRI/XboI-fragment into pGEX-5X-1 (Pharmacia).
DNA-oligonucleotides used were: GSP-I, 5'-

10 dTCCTGATGTTGCTGTAGACG-3',
GSP-II, 5'-dGCACGACTAGTATGATTTGC-3', and the 5'RACE
anchor primer (BRL), 5'-
dCUACUACUACUAGGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3'

15   7.1.2. IDENTIFICATION OF NS1-INTERACTORS
         The interactive trap selection was performed
essentially as described for NPI-1 in Section 6.1.2,
above. The selection strain was constructed by
transforming EGY48 with the bait plasmid pLexA-NS1 and

20 the lacZ-reporter plasmid pSH18-34. Expression of
lacZ from pSH18-34 is transcriptionally controlled by
a GAL1 promoter and LexA-dependent operator sites. A
HeLa cell cDNA library was introduced into the
selection strain using the lithium acetate method

25 (Ito, et al., 1983, supra). Primary transformants
were selected on trp⁻his⁻ura⁻ glucose plates. 1 x 10⁶
cells representing 3.3 x 10⁵ independent transformants
were plated on 150 mm trp⁻his⁻ura⁻leu⁻-galactose plates
to select for clones expressing NS1-interacting

30 proteins. Viable cells were replica-transferred to a
nitrocellulose filter and assayed for β-galactosidase
activity using 5-bromo-4-chloro-3-indolyl β-D-
galactoside (X-gal) as described (Ausubel et al.,
1992, supra). Positive clones were tested in a second

35

- 45 -

round of selection by replica plating onto X-gal trp⁻ his⁻ura⁻ galactose plates. Plasmid DNA was isolated from yeast clones expressing β-galactosidase activity only on galactose plates and library plasmids were recovered by transformation into *E.coli* MH3 as
5 described in Section 6.1.2, above. The specificity of the isolated plasmids was tested by co-transformation with pLexA-NS1 or pRFHM1 into EGY40 harboring pSH18-34. pRFHM1 expresses an unrelated LexA-bicoid fusion protein. The resulting strains were assayed for β-
10 galactosidase activity on X-gal trp⁻his⁻ura⁻ plates containing glucose or galactose. Plasmids that induced β-galactosidase only in the presence of galactose and only in conjunction with pLexA-NS1 were considered to encode true interacting proteins.
15

7.1.3. CLONING OF NS1I-1 5'-END CDNA

Cloning of cDNA derived from the 5'-end of NS1I-1 mRNA followed a RACE-procedure (rapid amplification of cDNA ends) (Frohmanm, et al., 1988,
20 *supra*) using a 5'RACE-kit (BRL). First strand cDNA was synthesized from 1 μg of HeLa cell poly(A)-RNA hybridized to 2.5 pmol NS1I-1-specific oligonucleotide GSP-I using reverse transcriptase. The cDNA was tailed at the 5'-end with dC by terminal transferase.
25 The product was used as a template for the amplification of a 5'RACE-product by PCR using a nested oligonucleotide GSP-II and an anchor primer provided by the kit. The resulting fragment was subcloned in pGEM-T (Promega) to form pRACENS1I-1, and
30 sequenced by the standard dideoxy method. The NCBI-search was conducted using Fasta, Tfasta. Sequence comparison was conducted using Bestfit.

7.1.4. NORTHERN BLOT ANALYSIS
35

● ●

1 µg of HeLa cell poly(A)-RNA was separated
on a 1% agarose-formaldehyde gel, transferred to a
nylon membrane (Nytran, Amersham), and UV-crosslinked.
The RNA was hybridized to a ^{32}P-labled, NS1I-1-specific
probe derived form a fragment (corresponding to
5 positions +791 to +1745) of the original pK5 library
isolate as described (Ausubel, et al., 1992, *supra*).

7.1.5. VIRUSES, CELLS, AND EXTRACTS

Influenza strains A/WSN/33 (H1N1),
10 A/Berkeley/1/68 (H2N2), A/Beijing/32/92 (H3N2),
A/duck/Alberta/76 (N12N5), A/turkey/Oregon/71 (H7N5),
and B/Lee/40 were grown in the allantoic cavity of
10 days old embryonated chicken eggs. Confluent
monolayers of Madin Darby canine kidney-(MDCK)-cells
15 were infected with influenza viruses at an m.o.i. of
10 for one hour in 35 mm dishes. Infection was
continued at 37°C (influenza A viruses) or 35°C
(influenza B/Lee/40) for 5 hours in MEM-medium
containing 0.1% bovine serum albumin. Cells were
20 labeled with 100 µCi of ^{35}S-methionine and ^{35}S-cysteine
(ICN) per dish for one hour in MEM-met-cys--medium.
Cells were washed and scraped in ice-cold phosphate
buffered saline (PBS). Cells from one dish were lysed
with 500 µl NET-N buffer (10mM Tris/HCL pH 8.0, 1 mM
25 EDTA, 150 mM Nacl, 0.05% Nonidet P 40) and two 30
second pulses in a Raytheon sonicator at a setting of
1A. Lysates were centrifuged for 10 minutes at 20,000
rpm in a TL100.3 rotor. The supernatants were used
for precipitation of proteins.
30

7.1.6. EXPRESSION OF GST-NS1I-1 FUSION PROTEIN IN E. COLI AND PRECIPITATION OF VIRAL PROTEINS FROM CELL EXTRACTS

NS1I-1 was expressed in *E. coli* BL26 from
35 pGEX-NS1I-1 as a GST (glutathione-S-transferase)-NS1I-

1 fusion protein with a predicted molecular weight of
77 kDa.   Production of GST-NS1I-1 was induced using
isopropyl-β-D-galactopyranoside essentially as
described (Smith, et al., 1988, *supra*). GST-NS1I-1
was adsorbed from bacterial lysates to glutathione

5 sepharose beads (Pharmacia) as recommended by the
manufacturer. Beads were washed three times with PBS
to remove contaminating proteins. 10 μl of
glutathione sepharose coated with GST-NS1I-1 fusion
protein was rotated with 100 μl extract of virus-

10 infected MDCK-cells (see above) in 750 μl NET-100
buffer (20 mM Hepes,pH 8.0, 100mM NaCl, 0.5 mM DTT)
for 90 minutes at 4°C. The beads were washed three
times with PBS/0.05% NP-40 and precipitated proteins
were analyzed by SDS-gel electrophoresis and

15 autoradiography. In parallel reactions, viral
proteins were immunoprecipitated from 50 μl of
infected cell extracts using 5 μl of anti-NS1 or anti-
M1 antiserum and protein A agarose as described
(Harlow & Lane, 1988, *supra*). As a negative control,

20 GST protein was expressed in BL26 from pGEX-5X-1 and
used the same way in the co-precipitation assay.

7.2.   RESULTS

25   7.2.1.   ISOLATION OF NS1-INTERACTING FACTORS
              The yeast interaction trap system (Gyuris,
et al., 1993, *supra*; Zervos, et al., 1993, *supra*) was
used to identify cellular proteins that interact with
the non-structural protein NS1 of influenza A virus.

30 A LexA-NS1 fusion protein was used as bait to screen
library in which HeLa cell cDNAs were expressed as
fusions with an acidic transcription activation domain
(Gyuris, 1993 #159). Colonies were selected, in which
either of two reporter genes, LEU2 and lacZ, were

35 activated by the cDNA-encoded proteins. This double

selection scheme was used to increase the stringency, because in an initial screen a high proportion of candidates scored negative in subsequent genetic tests. The library plasmids were isolated from the selected clones.

5   The binding specificity of the encoded fusion proteins was tested by assaying the activation of a lacZ-reporter gene encoded on pSH18-34. Expression of β-galactosidase from this plasmid is transcriptionally controlled by LexA-specific operator

10 sites. The isolated library plasmids were co-transformed with pLexA-NS1 or pRFHM1 into EGY40 harboring pSH18-34. pRFHM1 expresses a LexA-bicoid fusion protein and was used as a non-specific operator-binding control. The resulting strains were

15 assayed for β-galactosidase activity specifically on X-gal plates containing galactose, but not glucose. From 3.3×10^5 independent library transformants, three plasmids were isolated that induced galactose-specific activation of the lacZ reporter gene only in

20 combination with pLexA-NS1. Sequence analysis indicated that the three plasmids were each derived from different cellular cDNAs.

  7.2.2. CLONING AND SEQUENCE ANALYSIS OF NS1I-1

25   One of the isolated plasmids, pK5, was analyzed further. It carried a cDNA-insert of 1781 bp with an open reading frame of 1413 nucleotides followed by 368 nucleotides of a potentially untranslated region (Fig. 12). The cDNA terminated

30 with an oligo(A)-tract and had a consensus poly(A)-site at positions 2526-2531. Northern blot analysis of HeLa cell poly(A)-RNA using a NS1I-1-specific probe detected one single transcript of about 3.0 kb suggesting that the pK5 insert represented an

35 incomplete cDNA (Fig. 13). The remaining NS1I-1 cDNA

was cloned by a 5.'RACE procedure (Frohman, et al.,
1988, *supra*). Four independent clones were sequenced
that differed only in length at the very 5'-end. The
longest 5'RACE product, contained in pRACENS1I-1,
extended the NS1I-1 sequence for 893 nucleotides

5   upstream totalling in a cDNA of 2674 bp (Fig. 12).
The sequence has one long open reading frame encoding
a protein of 735 amino acids with a predicted
molecular mass of 79.5 kDa and a pI of 9.06. The
putative ATG-start codon is located 103 nucleotides

10 downstream of the 5'-end and is in the context of a
sequence consistent with its being a translational
start (Kozak, 1989, J. Cell Biol. 108: 229-241).
        Sequence comparisons through the EMBL- and
Genbank databases using the FASTA- and TFASTA-analysis

15 programs revealed that NS1I-1 is highly homologous to
porcine 17β-estradiol dehydrogenase (Leenders, et al.,
1994, *supra*). The two cDNAs are 86% identical on the
nucleic acid level. The encoded proteins are 84%
identical and are 92% similar when allowing for

20 conserved amino acid substitutions. NS1I-1 cDNA also
shows strong homology to ten human cDNA fragments that
have been isolated as expressed sequence tags, as
revealed by a BLAST-analysis of the NCBI-database
(fragments are between 134 to 556 bp in length).

25 These cDNAs were derived from different tissues
including liver, spleen, brain, adipose tissue, and
adrenals tissue indicating a broad expression of NS1I-
1 in the body.
        The encoded NS1I-1 protein features two

30 conserved sequence motifs of the short-chain alcohol
dehydrogenase family (Persson, et al., 1991, Eur. J.
Biochem. 200: 537-543). Specifically, amino acids 15
to 22 (TGAGAGCG) are similar to the potential co-
factor binding site, and residues 163 to 167 (YSAAK)

35 correspond to a short stretch that has been suggested

- 50 -

to participate in catalysis (Chen, et al., 1993, Biochemistry 32: 3342-3346). The presence of the tri-peptide AKL at the carboxy-terminus was also noted. Similar tri-peptide motifs have been found to serve as targeting signals for import into microbodies (for a
5   review, see de Hoop & Ab, 1992, Biochem. J. 286: 657-669). However, the presence of this signal does not automatically direct a protein to these organelles (de Hoop & Ab, 1992, *supra*).

10   7.2.3.   NS1I-1 BINDS NS1 PROTEIN FROM EXTRACTS OF
                 INFLUENZA VIRUS INFECTED CELLS
                 In order to confirm a physical interaction between NS1I-1 protein and NS1 expressed in influenza virus infected cells, a co-precipitation assay was
15 performed as similarly described in Section 6.2.3, above, for NPI-1. A glutathione-S-transferase(GST)-NS1I-1 fusion gene was constructed and expressed in *E.coli*. GST-NS1I-1 fusion protein from bacterial lysate was absorbed to the affinity matrix glutathione
20 agarose and purified from contaminating bacterial proteins. The immobilized fusion protein was used to bind and precipitate ^{35}S-labeled proteins from extracts of MDCK cells infected with human influenza A/WSN/33 viruses (Fig. 14). The NS1 protein of this strain is
25 98% identical to its counterpart from A/PR/8/34 used in the yeast interaction screen. Aliquots of the same extract were used to in parallel reactions to immunoprecipitate influenza virus proteins NS1 and M1. The precipitated proteins were analyzed by SDS-gel
30 electrophoresis and visualized by fluorography. Fig. 14 shows, that GST-NS1I-1 efficiently precipitated a protein band co-migrating with immunoprecipitated NS1 protein from infected cell extract (compare lanes 2 and 3). This interaction was specific for NS1I-1
35 since no proteins were detected in precipitates using

GST only (lane 6). In addition, no proteins were
precipitated by GST-NS1I-1 from mock-infected cells
(lane 8), showing that a virus induced protein was
recognized by NS1I-1. This experiment confirmed, that
NS1I-1 interacts specifically with the NS1 protein of
5 influenza A virus.

        If this interaction is important for the
viral life-cycle one would expect it to be conserved.
Consequently, the binding of NS1I-1 to NS1 proteins
from other influenza A strains should be detectable
10 despite of their considerable variation in the primary
structure (Baez, et al., 1981, Virology 113: 397-402;
Ludwig, et al., 1991, Virology 183: 566-577).
Therefore the interaction between NS1I-1 and NS1 was
examined using the same co-precipitation assay
15 described above, with extracts from cells infected
with different influenza A and B virus strains.

        Mutations accumulate in the NS1 gene at a
steady rate over time (Buonagurio, et al., 1985,
Science 232: 980-982). Thus, the time-span between
20 the isolation of two strains is reflected in the
sequence variation of its NS1 proteins (Ludwig, et
al., 1991, supra; Buonagurio, et al., 1985, supra).
NS1I-1 binding to NS1 proteins from two recently
isolated human influenza A strains A/Beijing/32/92 and
25 A/Berkeley/1/68 was examined. As can be seen in Fig.
15, Panels C and D, respectively, NS1 proteins from
both strains were specifically precipitated (Fig 15,
Panels C and D, lanes "GST-K5"). A low
immunoprecipitation efficiency of NS1 protein from the
30 Beijing-strain (Panel C) was reproducibly observed.
The NS1 proteins of A/Berkeley/1/68 and A/WSN/33 are
90.8% identical to each other. The NS1 sequence of
A/Beijing/32/92 is not known.

        The following analyses were conducted to
35 examine whether GST-NS1I-1 is also recognized by the

- 52 -

more divergent NS1 proteins of the avian influenza
strains A/duck/Alberta/76 and A/turkey/Oregon/71. The
NS1 proteins of these strains are 66.5% and 63.6%
identical, respectively, to A/WSN/33. Significantly,
NS1 of A/turkey/Oregon/71 is only 124 amino acids in
5 length, lacking most of the carboxy-terminal half of
other NS1 proteins, which consist of 207 to 237 amino
acids (Norton, et al., 1987, Virology 156: 204-213).
Nevertheless, precipitation of a protein band co-
migrating with NS1 from both strains was observed
10 (Fig. 15, Panels A and B, lanes "GST-K5"). The NS1
and M1 proteins of A/duck/Alberta/76 could not be
separated by the gel system used. Significant amounts
of nucleoprotein in the GST-NS1I-1 precipitates of
these avian strains were reproducibly detected for
15 undetermined reasons.

Finally, the co-precipitation assay was used
to test the human influenza B virus B/Lee/40.
Surprisingly, GST-NS1I-1 precipitated specifically the
influenza B virus NS1 protein, although it is only
20 20.6% identical to NS1 from A/WSN/33 (Fig. 15, Panel
E, lane "GST-K5"). Taken together, the binding of
GST-NS1I-1 to NS1 proteins expressed by several
influenza A and B virus stains could be demonstrated,
despite the great divergence of their primary
25 structures. This result strongly supports an
important function of this interaction during the
viral life cycle, and indicates that the NS1I-1
interaction is an excellent target for antiviral
intervention.
30 The present invention is not to be limited in
scope by the specific embodiments described which are
intended as single illustrations of individual aspects
of the invention, and functionally equivalent methods
and components are within the scope of the invention.
35 Indeed, various modifications of the invention, in

addition to those shown and described herein will
become apparent to those skilled in the art from the
foregoing description and accompanying drawings. Such
modifications are intended to fall within the scope of
the appended claims.

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MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page _, lines __ of the description '

A. IDENTIFICATION OF DEPOSIT '

Further deposits are identified on an additional sheet '

Name of depositary institution '

American Type Culture Collection

Address of depositary institution (including postal code and country) '

12301 Parklawn Drive
Rockville, MD 20852
US

Date of deposit ' <u>November 30, 1994</u> Accession Number ' <u>75950</u>

B. ADDITIONAL INDICATIONS ' (leave blank if not applicable). This information is continued on a separate attached sheet

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE ' (if the indications are not all designated States)

D. SEPARATE FURNISHING OF INDICATIONS ' (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later ' (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☐ This sheet was received with the International application when filed (to be checked by the receiving Office)

_____
(Authorized Officer)

☐ The date of receipt (from the applicant) by the International Bureau *

was   _____
(Authorized Officer)

· Form PCT/RO/134 (January 1981)

●

●

International Application No: PCT/   /

Form PCT/RO/134 (cont.)

American Type Culture Collection

12301 Parklawn Drive
Rockville, MD 20852
US

<u>Accession No.</u>

75951

<u>Date of Deposit</u>

November 30, 1994

WHAT IS CLAIMED IS:

1. An assay for identifying a substance that inhibits the specific interaction of a host cell protein, that is not a cell surface receptor protein, with a viral protein required for viral infection,
5 replication, assembly or release, comprising:

(a) contacting a protein or peptide containing an amino acid sequence corresponding to the binding site of the host cell protein with a protein or peptide having an amino acid sequence corresponding
10 to the binding site of the viral protein, under conditions and for a time sufficient to permit binding and the formation of a complex, in the presence of a test substance, and

(b) detecting the formation of a complex,
15 in which the ability of the test substance to inhibit the interaction between the host cell protein and the viral protein is indicated by a decrease in complex formation as compared to the amount of complex formed in the absence of the test substance.
20

2. An assay for identifying a substance that inhibits the interaction of influenza virus NP with a host cell protein comprising:

(a) contacting a protein or peptide
25 containing an amino acid sequence corresponding to the binding site of influenza virus NP with a protein or peptide containing an amino acid sequence corresponding to the binding site of the host cell protein, under conditions and for a time sufficient to
30 permit binding and formation of a complex, in the presence of a test substance, and

(b) detecting the formation of a complex, in which the ability of a test substance to inhibit the interaction between influenza virus NP and the host
35 cell protein is indicated by a decrease in complex

- 57 -

formation as compared to the amount of complex formed
in the absence of the test substance.

3.   The assay of Claim 2 in which the host cell
protein is NPI-1.

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4.   The assay of Claim 3 in which the host cell
protein is NPI-2.

5.   The assay of Claim 3 in which the host cell
10 protein is NPI-3.

6.   The assay of Claim 3 in which the host cell
protein is NPI-4.

15   7.   The assay of Claim 3 in which the host cell
protein is NPI-5.

8.   The assay of Claim 3 in which the host cell
protein is NPI-6.
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9.   An assay for identifying a substance that
inhibits the interaction of influenza virus NS1 with a
host cell protein comprising:
      (a)   contacting a protein or peptide
25 containing an amino acid sequence corresponding to the
binding site of influenza virus NS1 with a protein or
peptide containing an amino acid sequence
corresponding to the binding site of the host cell
protein, under conditions and for a time sufficient to
30 permit binding and formation of a complex, in the
presence of a test substance, and
      (b) detecting the formation of a complex, in
which the ability of a test substance to inhibit the
interaction between influenza virus NS1 and the host
35 cell protein is indicated by a decrease in complex

formation as compared to the amount of complex formed in the absence of the test substance.

10. The assay of Claim 9 in which the host cell protein is NS1I-1.

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11. The assay of Claim 1, 2, or 9 in which one protein or peptide of the complex is immobilized, and the other protein or peptide is labeled with a signal-generating compound.

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12. The assay of Claim 11 in which an immobilized antibody is used to anchor the immobilized protein or peptide.

15   13. The assay of Claim 11 in which a labeled antibody is used to label the protein or peptide with a signal-generating compound.

14. The assay of Claim 11 in which the protein
20   or peptide substrate is immobilized prior to the reaction so that the reaction is conducted in a solid-liquid phase.

15. The assay of Claim 1, 2, or 9 in which the
25   proteins or peptides are contacted in a liquid phase to form a complex which is separated from the liquid phase at the end of the reaction.

16. The assay of Claim 15, in which the complex
30   formed is separated from the liquid phase by immobilizing the complex on a solid phase.

17. The assay of Claim 16 in which the complex is captured by an immobilized antibody specific for
35   one of the proteins or peptide binding partners.

18.   A method for treating influenza virus
infection, comprising administering to an infected
individual, a therapeutically effective amount of a
substance that inhibits the specific interaction of a
host cell protein with a viral protein required for
5 viral infection, replication, assembly or release.

19.   The method of Claim 18 in which the viral
protein is NP.

10   20.   The method of Claim 18 the host cell protein
is NPI-1.

21.   The method of Claim 18 the host cell protein
is NPI-2.
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22.   The method of Claim 18 the host cell protein
is NPI-3.

23.   The method of Claim 18 the host cell protein
20 is NPI-4.

24.   The method of Claim 18 the host cell protein
is NPI-5.

25   25.   The method of Claim 18 the host cell protein
is NPI-6.

26.   The method of Claim 18 the viral protein is
NS1.
30

27.   The method of Claim 18 the host cell protein
is NS1I-1.

28.   An isolated DNA sequence which encodes the
35 amino acid sequence of NPI-1, or which selectively

hybridizes to the complement of the coding sequence of
NPI-1 and encodes a functionally equivalent gene
product.

29.   An isolated DNA sequence which encodes the
5 complement of the DNA sequence of Claim 28.

30.   A DNA vector containing the DNA sequence of
Claim 28.

10   31.   A DNA vector containing the DNA sequence of
Claim 29.

32.   An expression vector containing the DNA
sequence of Claim 28 operatively associated with a
15 regulatory element that directs the expression of the
DNA sequence.

33.   A genetically engineered host cell
containing the DNA sequence of Claim 28 operatively
20 associated with a regulatory element that directs
expression of the DNA sequence in the host cell.

34.   A DNA sequence which encodes the amino acid
sequence of NPI-2, NPI-3, NPI-4, NPI-5, or NPI-6 or
25 which selectively hybridizes to the complement of the
coding sequence of NPI-2, NPI-3, NPI-4, NPI-5, or NPI-
6 and encodes a functionally equivalent gene product.

35.   A DNA sequence which encodes the complement
30 of the DNA sequence of Claim 34.

36.   A DNA vector containing the DNA sequence of
Claim 34.

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37.   A DNA vector containing the DNA sequence of
Claim 35.

38.   An expression vector containing the DNA
sequence of Claim 34 operatively associated with a
5 regulatory element that directs the expression of the
DNA sequence.

39.   A genetically engineered host cell
containing the DNA sequence of Claim 34 operatively
10 associated with a regulatory element that directs
expression of the DNA sequence in the host cell.

40.   An isolated DNA sequence which encodes the
amino acid sequence of NS1I-1, or which selectively
15 hybridizes to the complement of the coding sequence of
NS1I-1 and encodes a functionally equivalent gene
product.

41.   An isolated DNA sequence which encodes the
20 complement of the DNA sequence of Claim 40.

42.   A DNA vector containing the DNA sequence of
Claim 40.

25   43.   A DNA vector containing the DNA sequence of
Claim 41.

44.   An expression vector containing the DNA
sequence of Claim 40 operatively associated with a
30 regulatory element that directs the expression of the
DNA sequence.

45.   A genetically engineered host cell
containing the DNA sequence of Claim 40 operatively
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associated with a regulatory element that directs
expression of the DNA sequence in the host cell.

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A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12Q 1/70; C12N 5/00, 15/00; A01N 37/18; C07H 21/04

US CL :435/5,240.2,320.1; 514/2; 536/23.5

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/5,240.2,320.1; 514/2; 536/23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Dialog

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	W. K. Joklik et al, "Zinsser Microbiology," published 1992 by Appleton and Lange (Norwalk, Connecticut), pages 854-861, see entire document.	1,2,11-17
A	Virus Research, Vol. 1, No. 6, issued September 1984, S. Nakada et al., "Complete nucleotide sequence of the influenza C/California/78 virus nucleoprotein gene," pages 433-441, see entire document.	1-8, 11-17, 38-39

☒	Further documents are listed in the continuation of Box C.	☐	See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 AUGUST 1995	29 AUG 1995

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer DONNA C. WORTMAN, Ph.D.
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document. with indication. where appropriate. of the relevant passages	Relevant to claim No.
A, P	The EMBO Journal, Vol. 13, No. 13, issued 1994, F. Baudin et al., "Structure of influenza virus RNP. I. Influenza virus nucleoprotein melts secondary structure in panhandle RNA and exposes the bases to the solvent," pages 3158-3165, see entire document.	1-8, 11-17, 28-39

INTERNATIONAL ●ARCH REPORT

Box I   Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II   Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

   Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
   1-8, 11-17, 28-39

Remark on Protest   ☐ The additional search fees were accompanied by the applicant's protest.

   ☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1))(July 1992)*

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined. the appropriate additional examination fees must be paid.

Group I. claims 1-8. 11-17. 28-39, drawn to a first product and assay.
Group II. claims 9-17, 40-45, drawn to a second product and assay.
Group III. claims 18-25, drawn to treatment method involving first product.
Group IV. claims 26, 27, drawn to treatment method involving second product.
The inventions listed as Groups I-IV do not relate to a single inventive concept under PCT Rule 13.1 because. under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: Groups I and II lack the same or corresponding special technical feature because the claims of Group II are drawn to a distinctly different product from the product of Group I. Groups I and III lack the same or corresponding technical features because the method of Group III is a distinctly different method from the method of Group I, requiring different method steps and reagents. Groups II and IV lack the same or corresponding special technical feature because the method of Group IV is a distinctly different method from the method of Group II requiring different method steps and reagents. PCT Rule 13.2 does not provide for multiple products or methods.